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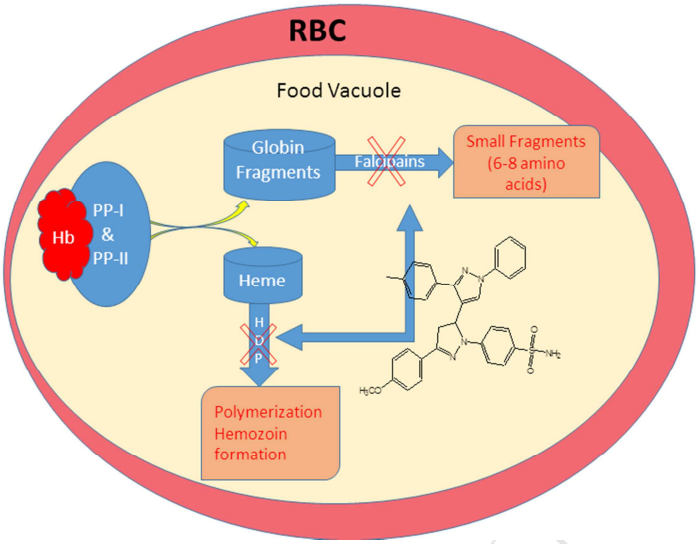

Pyrazole-Pyrazoline as Promising Novel Antimalarial Agents: A Mechanistic Study

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ABSTRACT:

A series of pyrazole-pyrazoline substituted with benzenesulfonamide were synthesized and evaluated for their antimalarial activity *in vitro* and *in vivo*. The compounds were active against both chloroquine (CQ) sensitive (3D7) and CQ resistant (RKL-9) strains of *Plasmodium falciparum*. Seven compounds (**7e, 7i, 7j, 7l, 7m, 7o** and **7p**) exhibiting EC_{50} less than 2μM. A mechanistic study of compound **7o** revealed that these compound act through the inhibition of β-hematin. The study indicated that these compounds can serve as lead compounds for further development of potent antimalarial drugs.

Key words: *Plasmodium falciparum*, antimalarial, β-hematin, heme binding, pyrazole, pyrazoline

1. Introduction

Malaria remains one of the most challenging infectious diseases in the world especially in third world and developing countries like India. Due to the development of resistance to the existing drug regimen the problem has increased too many folds [1]. Identification and development of new antimalarial agents specifically against new validated targets are therefore an urgent requirement [2]. Among the potential new targets for the development of antimalarial drugs directed against *P. falciparum* are proteases. For protein synthesis, erythrocytic malaria parasites acquire amino acids by degradation of haemoglobin. Multiple enzymes appear to participate in this process [3, 4] including the cysteine proteases falcipain-2 [5] and falcipain-3 [6]. The cysteine protease inhibitors have been shown to arrest the erythrocytic life cycle of *P. falciparum*, by blocking the hydrolysis of the host hemoglobin, causing abnormally swollen food vacuoles and also some of these molecules have led to the cure of malaria in mice. [7, 8].

One of the most widely explored pathways for development of antimalarial agent includes inhibition of hemozoin/β-hematin formation. Parasite converts toxic heme into a non-toxic hemozoin by a mechanism known as hemozoin formation and this process is considered to be the most important mechanism for heme detoxification in Plasmodium sp. [9]. Several types of antimalarial agents are reported to exhibit antimalarial activity by enhancing free heme toxicity through the inhibition of hemozoin formation. Compounds that show antimalarial activity via inhibition of hemozoin are presented in figure 1. Benzimidazole derivatives (1) [10], known antifungal agent miconazole (2) [11], clotrimazole (3) [12], dibromocryptolepine (4) [13], floxacrine (5) [14], along with other compounds like RO 22-8014 (6) [15], N1-(4-(benzyloxy)benzyl)-N3-(1-(2,2-diphenylethyl)piperidin-4-yl)propane-1,3-diamine (7), [14] and pyrazole (8) [16] are reported as inhibitors of hemozoin formation. Efforts, therefore, continue to discover newer antimalarial agents through different mechanisms and targets. We here in present the potential of pyrazole-pyrazoline based compounds for their antimalarial activity through β-hematin binding studies.

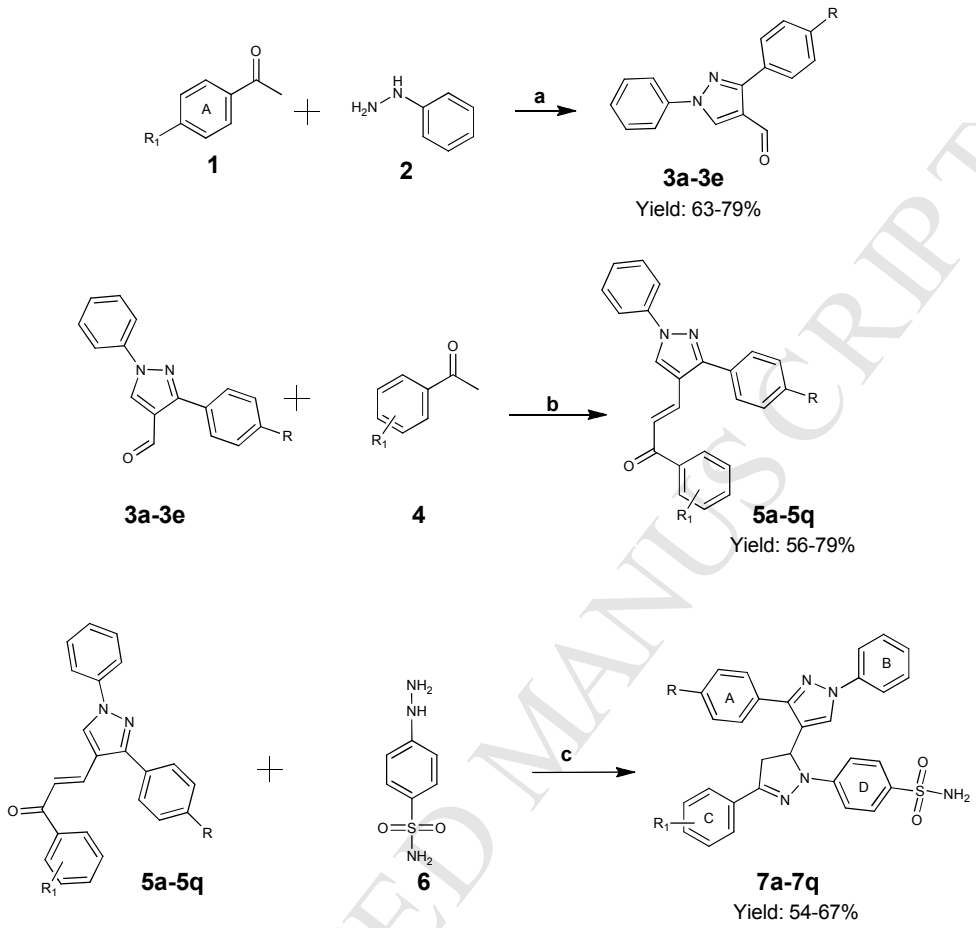
2. RESULTS AND DISCUSSION

The title compounds were synthesized successfully and their structure was elucidated on the basis of spectral analysis. All the synthesized compounds (**7a-q**) were tested for their antimalarial activity and most active compound was selected for the mechanistic study.

2.1.Chemistry

The synthesis of title compounds was carried out by the method outlined in Scheme 1. Pyrazole carbaldehydes (**3a-3e**) were synthesized by reported method [17] in 63-79% yield. The aldehydes (**3a-3e**) were made to react with substituted acetophenone by Claisen-Schmidt reaction in presence of sodium hydroxide in ethanol to obtain different chalcones (**5a-5q**) with 56-79% of yield. The bipyrazol-benzenesulfonamide derivatives (**7a-7q**) were prepared by cyclocondensation reaction of chalcones (**5a-5q**) with 4-hydrazinylbenzenesulfonamide in presence of HCl in ethanol with a yield of 54-67%. The compounds (**7a-7q**) were obtained as racemic mixtures and were fully characterized by IR, NMR, and MS. The IR spectra absorption bands at 3429-3335 cm^{-1} and 3292-3247 cm^{-1} supported presence of primary NH_2, absorption bands in the region of 1592-1596 cm^{-1} and 1491-1503 cm^{-1} indicated azomethine C= N and aromatic C=C stretching. The ^{1}H NMR spectra showed peak at 8.25-8.38 ppm which could be assigned to pyrazole protons whereas pyrazoline stereogenic proton and methylenic protons both couple and appear as double-doublets at (5.73-5.60) ppm, with $J_{5'-4a'}$=11.6-12.2 Hz, and $J_{5'-4b'}$=6.0- 6.8 Hz, and methylenic protons at δ= 4.02-4.18 ppm with $J_{4a'-4b'}$=12.4-17.4 Hz, and, $J_{4a'-5'}$=12-13.2 Hz, and 4b'-H at δ=3.30-3.36 ppm, $J_{4b'-4a'}$=16-17.6 Hz, and $J_{4b'-5'}$=6-6.4 Hz which indicates '*trans*' conformation. The $-NH_2$ peak could be located between 6.95-7.02 ppm. The ^{13}C NMR spectra show the absence of carbonyl carbon peak and presence of pyrazoline chiral carbon at 55.20-55.90 ppm, and pyrazoline methylenic carbon at 42.40-45.37 ppm. The mass spectra of the compound shows M^+, $(M-H)^+$, (M+2) peaks respectively. The synthesized compound were found pure beyond 95% by HRMS and HPLC data (Supplimentray data). Compound 7q showed two peaks of slight difference in height and retention time indicating that it may be present in recemic mixture. The X-ray crystallographic studies of the compound **7e** confirmed that the conformation of the compound at chiral centre is '*trans*' (Fig. 2). Crystallographic data for the structure **7e** have been deposited with the Cambridge Crystallographic Data Center (CCDC) under the number CCDC- 1475330.

Scheme 1: Synthesis of 4-(5-(1,3-diphenyl-1H-pyrazol-4-yl)-3-phenyl-4,5-dihydropyrazol-1-yl) benzenesulfonamide Derivatives (7a-7q)



Reagents and conditions: (a) POCl3, DMF; NaHCO3, reflux; (b) MeOH, NaOH, reflux; (c) MeOH, HCl, reflux

2.2.Biological activity

2.2.1. In vitro and in vivo antimalarial activity

Compounds (**7a -7q**) were evaluated for their *in vitro* antimalarial activity against chloroquine CQ^S3D7 and CQ^RRKL-9 *pf* strain by reported method [16, 18] and *in vivo* antimalarial activity by Peter's 4-day suppressive test [16, 19] by using *P. berghei* NK65 strain. Cytotoxicity was also determined in VERO cells by reported procedure [20].The

results of *in vitro* antimalarial activity against CQ^S 3D7 and CQ^R RKL-9 strains of *P. falciparum* of synthesized compounds (**7a -7q**) is presented in Table 1. Most of the final compounds showed potent antimalarial activity against CQ^S 3D7 with seven compounds (**7e**, **7i**, **7j**, **7l**, **7m**, **7o** and **7p**) exhibiting EC_{50} less than 2 μM which is better than CQ. These compounds also exhibited good activity against CQ^RRKL-9 strains (EC_{50} 1.31 -2.12 μM) compared to the reference drug (EC_{50} 11.25 μM). Compounds exhibited good tolerance as indicated by SI index. Most potent compound **7o** was selected for *in vivo* antimalarial studies against *P. berghei* mouse model. The results obtained were encouraging as the parasitemia suppression of 82.49% was observed at a dose 50 mg/kg body weight on day 4 and the mean survival days were 19.8 days compared to 7.2 days for the negative control group (Table 2). Results are expressed as mean ± SD, Statistical evaluation was performed using ANOVA followed by Dunnet test for comparison against standard.

2.2.2. Mechanistic studies: hematin binding studies

Pyrazole based compounds are reported to show antimalarial activity from our lab earlier also [21, 22]. Also in our previous work reported pyrazole based compounds were found to act through the inhibition of β-hematin [16] therefore to draw a plausible mechanism for the synthesized compounds, compounds **7o** was selected for mechanistic. The hematin binding studies of compound **7o** was performed at pH 7.4, 5.6 and 5.8 (pH of food vacuole). An absorption band (Soret band) at 402 nm indicated the presence of hematin (FP-Fe(III)), under the conditions used (0.02 M HEPES buffer, pH 7.4 and 0.02 M MES buffer, pH 5.6). On addition of increased concentration of **7o** into a constant concentration of monomeric heme (5.0 μM), a decrease in the intensity of the Soret band was observed with no shift in the absorption maxima (Fig. 3). The stoichiometry ratio of the most stable complexes of compound **7o** with hematin at pH 5.6 and 7.4 was inferred from the Job's plot and was found to be 1:1. The absorbance at 402 nm got to maximum when mole fraction of the compound was approximately 0.5. Thus 1:1 ratio was established for the association of compound hematin at both the pH values (Fig. 4). Since CQ and its derivatives also bind to heme dimer, the study of the binding of compound **7o** with the heme dimer at pH 5.8 the physiological pH of plasmodium food vacuole was also studied. In aqueous NaOH solution, heme exists as dimer and shows an absorption band at 362 nm. The addition of compound **7o** (0−20 μM) to a solution of the dimer (10 μM) in 20 mM phosphate buffer at pH 5.8 resulted in a decrease

in intensity of absorbance (Soret band) at 362 nm. The Job's plot indicated a 1:1 stoichiometry for the most stable complex formed between heme dimer and compound **7o** (Fig. 5). From the data shown in Table 3, it is apparent that the compound **7o** binds strongly with monomeric heme as well as with heme dimer and the observed results are comparable to the standard CQ (log K 5.58). Thus the formation of complexes between soluble hematin and compound **7o** suggests the inhibition of formation of β-hematin, as one of the possible mechanisms of antimalarial action of these compounds. The hematic binding studies indicated that it is not the only mode of action of these derivatives as the antimalarial activity is excellent there must be another mode of action.

2.2.3. **Structure activity relationship (SAR)**

Pyrazole pyrazoline based compounds resulted in potent antimalarial agents against both chloroquine sensitive and chloroquine resistant stains of *P. falciparum*. From the activity of the series it could be observed that compounds with electron donating group like $–OCH_3$, CH_3, OH, on *para* position of ring C were favourable for activity. For example the most potent compound contained either $–OCH_3$, CH_3 (compound **7o** and **7e**). On ring A, no substitution or small electron donating group on *para* position were favourable for the activity. It was observed that the highest activity was shown by compound (**7o**) with CH_3 group on ring A rather than $–OCH_3$ group and the reason for this may increase in bulk of $–OCH_3$ group. This SAR is in agreement with the literature reports about the SAR of antimalarial compounds.

3. Conclusion:

The syntheses of new pyrazolo-pyrazoline substituted compounds (**7a-q**) were successfully attained and all of them were characterized by spectral analysis. All the compounds showed antimalarial activity against CQ^S (3D7) and selected compounds were also found active against CQ^R (RLK-9) strain of *P. falciparum.* Five compounds (**7e, 7i, 7m, 7o, 7p**) showed comparable activity compared to chloroquine in CQ^S strain and were found better than CQ^R Plasmodium strain. *In vivo* screening of compound **7o** in mouse model of malaria also showed promising results with good mean survival days. Compound **7o** also showed good inhibition of hematin at both the pH studied. The compounds seem to act through inhibition of β-hematin in addition to other unknown mechanisms. These results indicate that the title

compounds are promising lead for development of activity based agents and provide a significant model for further structural and biological optimization.

4. Experimental Section

4.1.Chemistry

All chemicals were purchased from commercial suppliers. Melting points were determined by an open capillary method by digital melting point apparatus by VEEGO and are uncorrected. ^{1}H NMR spectra were recorded on a Bruker Avance 400MHz spectrometer, using $CDCl_3$ and DMSO-d_6 as solvent and TMS as internal standard.The ^{13}C NMR spectra of compounds were recorded on Bruker Avance-100 MHz instrument in DMSO-d_6. The chemical shift in NMR is reported as delta (δ) values. Infrared (IR) spectra were recorded on a Shimadzu FT-IR 8400S infrared spectrophotometer using the ATR accessory. MS analysis was performed on an Agilent 6410 LC-MS (Agilent Technologies, USA) triple-stage quadrupole mass spectrometer equipped with electrospray ionization (ESI) interface. Electrospray ionization (ESI) was run in following operating conditions: Source and Mode; ESI, Polarity; positive and negative, Collision gas; nitrogen, Capillary voltage; ±3000 V, Ion source temperature; 3000C, Gas flow; 10L/min, Dwell time; 10ms, Resolution (Q1 and Q3); unit. High Resolution Mass Spectra (HRMS) were recorded on Agilent Technologies 6540 instrument. Reaction progress was monitored with the help of pre-coated TLC plates [0.25 mm Merck silica-gel plates (60 F254)] and spots were visualized under UV light and by exposing them to iodine vapors. The reactant pyrazolecarbaldehyde (**3a-3e**) was prepared by reported method. [19] These were then reacted to substituted acetophenone by Claisen Schmidt reaction to obtain chalcones which were cyclized to final pyrazoline derivatives by treating with 4-hydrazinylbenzenesulfonamide. The purity of the data was confirmed by HRMS and HPLC data. HPLC was performed on Shimadzu CLASS-VP V6.14 SP1 using HPLC grade acetonitrile and methanol in 9:1 ratio.

4.2. **General procedure for preparation of pyrazole carbaldehyde (3a-3e)**

Cooled $POCl_3$ (0.075 mole) was added dropwise through dropping funnel to previously cooled DMF (0.075 mole at 0-5°C) and stirred for 15 minutes. A solution of hydrazone (0.025 mole) in DMF was added drop-wise to the DMF- $POCl_3$ mixture. The mixture was then warmed to room temperature and heated at 75-80°C for 5 hour. The completion of

reaction was monitored with the help of TLC. After the completion of reaction the mixture was cooled to room temperature and basified with saturated solution of $NaHCO_3$. The precipitate was filtered, thoroughly washed with distilled water, dried and crystallized in the ethanol

4.2.1. *1,3-diphenyl-1H-pyrazole-4-carbaldehyde (3a)* white crystal (74%): mp: 122°C; ^{1}H NMR (400MHz, $CDCl_3$): δ=10.03 (s, 1H), 8.52 (s, 1H), 7.83-7.80 (m, 2H), 7.78-7.75 (m, 2H), 7.51-7.43 (m, 5H), 7.38 ppm (t, *J*=6.84 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ=119.69, 122.49, 127.92, 128.74, 128.95, 129.27, 129.64, 131.02, 131.33, 138.98, 154.70, 185.14 ppm; IR (KBr): $\tilde{\nu}$== 2836, 1671, 1596, 1525 Cm^{-1}.

4.2.2. *3-(4-fluorophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (3b)* white crystal (67%): mp: 150°C; ^{1}H NMR (400MHz, $CDCl_3$): δ=10.03 (s, 1H), 8.53 (s, 1H), 7.88-7.85 (m, 2H), 7.80-7.77 (m, 2H), 7.54 (t, *J*=7.44 Hz, 2H), 7.42 (t, *J*=7.4 Hz, 1H), 7.21 ppm (t, *J*=7.6 Hz, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ=115.67, 115.88, 119.74, 122.43, 127.49, 127.52, 128.07, 129.73, 130.77, 130.85, 131.81, 138.94, 153.49, 162.28, 164.75, 184.62 ppm; IR (KBr): $\tilde{\nu}$==2837, 1672, 1597, 1522 Cm^{-1}.

4.2.3. *3-(4-chlorophenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (3c)* white crystal (79%): mp: 124°C; ^{1}H NMR (400MHz, $CDCl_3$): δ=10.03 (s, 1H), 8.52 (s, 1H), 7.84-7.82 (m, 2H), 7.81-7.76 (m, 2H), 7.53-7.42 (m, 4H), 7.42-7.39 ppm (m, 1H); ^{13}C NMR (100 MHz, $CDCl_3$) δ= 119.72, 122.50, 128.11, 128.93, 129.74, 129.84, 130.19, 132.03, 135.43, 138.88, 153.18, 184.46 ppm; IR (KBr): $\tilde{\nu}$==2830, 1672, 1600, 1521 Cm^{-1}.

4.2.4. *1-phenyl-3-(p-tolyl)-1H-pyrazole-4-carbaldehyde (3d)* white crystal (71%): mp: 99°C; ^{1}H NMR (400MHz, $CDCl_3$): δ=10.04 (s, 1H), 8.52 (s, 1H), 7.79-7.77 (m, 2H), 7.72 (d, *J*=7.24 Hz, 2H), 7.51 (t, *J*=6.52 Hz, 2H), 7.40 (t, *J*=6.68 Hz, 1H), 7.31 (d, *J*=7.92 Hz, 2H), 2.46 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) $\delta_C$ppm 21.38, 119.74, 122.46, 127.90, 128.46, 128.85, 129.48, 129.66, 130.87, 139.05, 139.35, 154.89, 185.34 ppm; IR (KBr): $\tilde{\nu}$==2834, 1671, 1596, 1518 Cm^{-1}.

4.2.5. *3-(4-methoxyphenyl)-1-phenyl-1H-pyrazole-4-carbaldehyde (3e)* white crystal(63%): mp: 118°C; ^{1}H NMR (400MHz, $CDCl_3$): δ=10.03 (s, 1H), 8.51 (s, 1H), 7.80-7.76 (m, 4H), 7.51 (t, *J*=6.48 Hz, 2H), 7.39 (t, *J*=7.36 Hz, 1H), 7.03 (d, *J*=6.68 Hz, 2H), 3.86 ppm (s, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ=55.37, 114.19, 119.68, 122.35, 123.84, 127.85, 129.65,

130.26, 131.22, 139.04, 154.46, 160.54, 185.15 ppm; IR (KBr): $\tilde{\nu}$==2854, 1671, 1595, 1525 Cm^{-1}.

*4.3.General procedure for preparation of bipyrazol-benzenesulfonamide (**7a-7q**)*

To a mixture of ethanol (35 ml) and 0.5 ml HCl, chalcones (0.001 mol) and 4-hydrazinylbenzenesulfonamide (0.0025 mol) were added. The mixture was stirred vigorously at 75°C and the completion ofthe reaction was monitored with the help of TLC. After completion of the reaction, the reaction mixture was cooled to room temperature and quenched by pouring the mixture into the crushed ice. The icy mixture was neutralized with $NaHCO_3$ solution to get the precipitated product. The precipitate was filtered, dried using a vacuum and crystallized in the mixture of ethanol and chloroform (1: 0.25).

4.3.1. *4-(5-(2,4-dichlorophenyl)-1',3'-diphenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7a**):*

Yellow crystal (60%): mp: 200°C; ^{1}H NMR (400MHz, DMSO) δ=8.38 (s, 1H), 7.82 -7.77 (m, 3H), 7.71 (t, *J*=7.6 Hz, 3H), 7.58 (d, *J*=8.8 Hz, 2H), 7.50-7.41 (m, 7H), 7.28 (t, *J*=7.6 Hz, 1H), 7.04 (s, 1H), 7.02 (s, 2H), 5.73 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.18 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.45 ppm (dd, *J*=17.4 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=45.3, 55.9, 112.9, 118.8, 121.5, 126.3, 126.7, 127.3, 127.5, 128.1, 128.5, 128.9, 129.5, 129.6, 130.6, 131.1, 132.7, 132.8, 133.9, 134.8, 139.5, 146.0, 147.1, 150.3 ppm; MS m/z 588.00 $(M+H)^+$, 590.09 $(M+2)^+$; IR (KBr): $\tilde{\nu}$=3423, 3292, 1592, 1502 Cm^{-1}.

4.3.2. *4-(5-(4-chlorophenyl)-1',3'-diphenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7b**)*

White crystal (64%): mp: 198°C; ^{1}H NMR (400MHz, DMSO) δ= 8.33 (s, 1H), 7.82 (d, *J*=7.6 Hz, 2H), 7.77 (t, *J*=8.8 Hz, 4H), 7.57 (d, *J*=8.8 Hz, 2H), 7.51 (t, *J*=7.6 Hz, 4H), 7.44 (t, *J*=7.6 Hz, 3H), 7.27 (t, *J*=7.2 Hz, 1H), 7.04 (d, *J*=8.8 Hz, 2H), 6.96 (s, 2H), 5.71 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.08 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.36 ppm (dd, *J*=17.6 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.4, 55.0, 112.9, 118.2, 121.4, 126.4, 127.0, 127.1, 127.73, 128.02, 128.29, 128.69, 129.45, 130.80, 132.53, 133.49, 133.63, 139.12, 145.89, 148.69, 149.86 ppm; MS m/z 554.00 (M+H), 555.17 (M+2) ; IR (KBr): $\tilde{\nu}$=3363, 3247, 1594, 1491 Cm^{-1}.

4.3.3. *4-(5-(4-fluorophenyl)-1',3'-diphenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7c**)*

Yellow crystal (58%): mp: 210°C; ^{1}H NMR (400MHz, DMSO) δ= 8.33 (s, 1H), 7.82-7.69 (m, 6H), 7.60 (d, *J*=8.8 Hz, 2H), 7.52 (t, *J*=7.2 Hz, 2H), 7.44 (t, *J*=8 Hz, 3H), 7.28 (t, *J*=8.8 Hz, 3H), 7.05 (d, *J*=8.8 Hz, 2H), 6.99 (s, 2H), 5.69 (dd, *J*=12.2 Hz, *J*=6.4 Hz, 1H), 4.08 (dd, *J*=15.4 Hz, *J*=12.4 Hz, 1H), 3.37 ppm (dd, *J*=14 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.64, 55.26, 111.98, 112.19, 114.10, 115.53, 115.74, 118.22, 122.03, 122.20, 124.43, 126.39, 127.07, 127.11, 127.70, 128.03, 128.23, 128.29, 128.53, 128.71, 129.45, 132.56, 132.92, 133.33, 139.14, 146.10, 146.34, 148.90, 149.73, 149.87, 161.41, 163.87 ppm; MS m/z 536.10 $(M-H)^{+}$; IR (KBr): $\tilde{\nu}$=3429, 3279, 1594, 1501 Cm^{-1}. HRMS m/z: 538.1577

4.3.4. *4-(5-(4-hydroxyphenyl)-1',3'-diphenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7d**)*

Yellow crystal (58%): mp: 264°C; ^{1}H NMR (400MHz, DMSO) δ=9.81 (s, 1H), 8.27 (s, 1H), 7.82 (d, *J*=8 Hz, 2H), 7.79 (d, *J*=7.6 Hz, 2H), 7.62 (d, *J*=8.4 Hz, 2H), 7.57 (d, *J*=8.8 Hz, 2H), 7.53 (t, *J*=7.2 Hz, 2H), 7.45-7.40 (m, 3H), 7.27 (t, *J*=7.2 Hz, 1H), 7.01 (d, *J*=8.8 Hz, 2H), 6.95 (s, 2H), 6.84 (d, *J*=8.4 Hz, 2H), 5.62 (dd, *J*=11.8 Hz, *J*= 6.4 Hz, 1H), 4.05 (dd, *J*=17 Hz, *J*=12.4 Hz, 1H), 3.31 ppm (dd, *J*=17.4 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.85, 54.99, 111.88, 115.50, 118.21, 122.27, 122.86, 126.37, 126.96, 127.05, 127.85, 127.98, 128.27, 128.73, 129.45, 132.59, 132.71, 139.15, 146.44, 149.80, 150.09, 158.75 ppm; MS m/z 534.00 (M-H); IR (KBr): $\tilde{\nu}$=3483, 3335, 3243, 1595, 1503 Cm^{-1}.

4.3.5. *4-(5-(4-methoxyphenyl)-1',3'-diphenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7e**)*

Yellow crystal (61%): mp: 192°C; ^{1}H NMR (400MHz, DMSO) δ=8.30 (s, 1H), 7.82 (d, *J*=8 Hz, 2H), 7.77 (d, *J*=7.2 Hz, 2H), 7.71 (d, *J*=8.8 Hz, 2H), 7.55 (t, *J*=8.8 Hz, 2H), 7.50 (d, *J*=7.6 Hz, 2H), 7.45-7.39 (m, 3H), 7.26 (t, *J*=7.6 Hz, 1H), 6.99 (d, *J*=9.2 Hz, 6H), 5.63 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.07 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.77 (s, 3H), 3.33 ppm (dd, *J*=17.2 Hz, *J*=6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=43.28, 55.51, 55.76, 112.44, 114.60, 118.68, 122.70, 124.89, 126.90, 127.55, 128.22, 128.80, 129.98, 133.04, 133.31, 139.61, 146.77, 150.24, 150.27, 160.70 ppm; MS m/z 550.20 $(M+H)^{+}$; IR (KBr): $\tilde{\nu}$=3382, 3258, 1592, 1502 Cm^{-1}.

4.3.6. *4-(5-(4-bromophenyl)-3'-(4-fluorophenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7f**)*

White crystal (76%): mp: 204°C; ^{1}H NMR (400MHz, DMSO) δ=8.35 (s, 1H), 7.81 (d, *J*=8 Hz, 2H), 7.75-7.71 (m, 2H), 7.68 (d, *J*=8.4 Hz, 2H), 7.63 (d, *J*=8.4 Hz, 2H), 7.57 (d, *J*=8.8 Hz, 2H), 7.43 (t, *J*=7.2 Hz, 2H), 7.31-7.23 (m, 3H), 7.04 (d, *J*=8.8 Hz, 2H), 6.99 (s, 2H), 5.70 (dd, *J*=12 Hz, *J*=6 Hz, 1H), 4.05 (dd, *J*=17.2 Hz, *J*=13.2 Hz, 1H), 3.34 ppm (dd, *J*=15.4 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.70, 55.69, 112.82, 115.97, 116.19, 118.71, 122.34, 122.86, 126.96, 127.57, 127.77, 128.45, 129.51, 129.98, 130.66, 130.74, 131.58, 132.09, 134.00, 139.56, 146.31, 149.28, 149.46 ppm; MS m/z 617.90 $(M+H)^{+}$, 618.24 $(M+2)^{+}$; IR (KBr): $\tilde{\nu}$=3367, 3251, 1595, 1498 Cm^{-1}. HRMS m/z: 616.0772

4.3.7. *4-(3',5-bis(4-fluorophenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7g**)*

White crystal (68%): mp: 212°C; ^{1}H NMR (400MHz, DMSO) δ=8.35 (s, 1H), 7.81 (m, 6H), 7.57 (d, *J*=8.8 Hz, 2H), 7.44 (t, *J*=7.6 Hz, 2H), 7.32-7.24 (m, 5H), 7.04 (d, *J*=8.8 Hz, 2H), 7.00 (s, 2H), 5.68 (dd, *J*=12 Hz, *J*= 6.4 Hz, 1H), 4.06 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.34 ppm (dd, *J*=17.4 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.98, 55.61, 112.69, 115.98, 116.05,116.19, 116.27, 118.70, 122.43, 126.95, 127.57, 128.72, 128.81, 128.97, 129.52, 129.99, 130.65, 130.74, 133.76, 139.56, 146.52, 149.43, 149.46, 161.39, 161.90, 163.83, 164.35 ppm; MS m/z 556.12 $(M+H)^{+}$;IR (KBr): $\tilde{\nu}$=3370, 3257, 1596, 1502 Cm^{-1}. HRMS m/z: 555.1579

4.3.8. *4-(3'-(4-fluorophenyl)-1'-phenyl-5-(p-tolyl)-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7h**)*

White crystal (62%): mp: 224°C; ^{1}H NMR (400MHz, DMSO) δ=8.32 (s, 1H), 7.81(d, *J*=8.0 Hz, 2H), 7.77-7.74 (m, 2H), 7.65 (d, *J*=8.4 Hz, 2H), 7.56 (d, *J*=8.8 Hz, 2H), 7.43 (t, *J*=7.6 Hz, 2H), 7.32 (t, *J*=8.8 Hz, 2H), 7.25 (t, *J*=8.0 Hz, 3H), 7.02 (d, *J*=8.8 Hz, 2H), 6.96 (s, 2H), 5.65 (dd, *J*=12.2 Hz, *J*=6 Hz, 1H), 4.05 (dd, *J*=17.2 Hz, *J*=12.00 Hz, 1H), 3.30 (dd, *J*=16.00 Hz, *J*=6.4 Hz, 1H), 2.46 ppm (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ=21.48, 55.48, 112.61,115.99, 116.20, 118.71, 122.54, 126.94, 127.56, 129.72, 129.98, 130.62, 130.70, 133.60, 139.46, 139.57, 150.37 ppm; MS m/z 550.00 $(M-H)^{+}$; IR (KBr): $\tilde{\nu}$=3369, 3253, 1596, 1502 Cm^{-1}.

4.3.9. *4-(3'-(4-fluorophenyl)-5-(4-hydroxyphenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7i**)*

White crystal (63%): mp: 228°C; ^{1}H NMR (400MHz, DMSO) δ=9.82 (s, 1H), 8.29 (s, 1H), 7.81-7.75 (m, 4H), 7.59 (d, *J*=8.8 Hz, 2H), 7.55 (d, *J*=8.8 Hz, 2H), 7.44 (t, *J*=7.6 Hz, 2H), 7.32-7.27 (m, 3H), 7.00 (d, *J*=8.8 Hz, 2H), 6.95 (s, 2H), 6.81 (d, *J*=8.8 Hz, 2H), 5.60 (dd, *J*=11.8 Hz, *J*=6.4 Hz, 1H), 4.02 (dd, *J*=17.2 Hz, *J*=12 Hz, 1H), 3.28 ppm (dd, *J*=17.4 Hz, *J*=6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=43.21, 55.37, 112.38, 115.99, 116.21, 118.71, 122.62, 123.29, 126.92, 127.55, 128.33, 129.54, 129.57, 129.98, 130.60, 130.68, 133.20, 139.58, 146.88, 149.40, 150.60, 159.23, 161.38 ppm; MS m/z 552.00 $(M-H)^{+}$.; IR (KBr): $\tilde{\nu}$=3466, 3401, 3292, 1594, 1501 Cm^{-1}.

4.3.10. *4-(3'-(4-fluorophenyl)-5-(4-methoxyphenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7j**)*

Yellow crystal (57%): mp: 170 °C; ^{1}H NMR (400MHz, DMSO) δ=8.31 (s, 1H), 7.81-7.74 (m, 4H), 7.70 (d, *J*=8.8 Hz, 2H), 7.55 (d, *J*=8.8 Hz, 2H), 7.43 (t, *J*=7.6 Hz, 2H), 7.32-7.23 (m, 3H), 7.00-6.95 (m, 6H), 5.62 (dd, *J*=11.8 Hz, *J*=6 Hz, 1H), 4.05 (dd, *J*=17.4 Hz, *J*=12 Hz, 1H), 3.78 (s, 3H), 3.30 ppm (m, 1H); ^{13}C NMR (100 MHz, DMSO) δ= 43.00, 55.42, 55.78, 112.47, 114.60, 116.00, 116.21, 118.69, 122.60, 124.85, 127.56, 128.20, 129.99, 130.61, 130.70, 150.26, 160.71 ppm; MS m/z 568.10 $(M+H)^{+}$; IR (KBr): $\tilde{\nu}$=3370, 3261, 1595, 1503 Cm^{-1}. HRMS m/z: 568.1776

*4.3.11. 4-(3',5-bis(4-chlorophenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7k**)*

Yellow crystal (70%): mp: 232 °C; ^{1}H NMR (400MHz, DMSO) δ=8.34 (s, 1H), 7.81 (d, *J*=8 Hz, 2H), 7.75-7.72 (m, 4H), 7.58 (d, *J*=9.2 Hz, 2H), 7.53-7.47 (m, 4H), 7.44 (t, *J*=8 Hz, 2H), 7.28 (t, *J*=7.2 Hz, 1H), 7.06 (d, *J*=8.8 Hz, 2H), 6.99 (s, 2H), 5.73 (dd, *J*=12.2 Hz, *J*=6 Hz, 1H), 4.06 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.34 ppm (dd, *J*=16 Hz, *J*=6 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=42.75, 55.72, 112.84, 118.77, 122.55, 127.04, 127.58, 127.89, 128.23, 129.21, 129.99, 130.27, 131.22, 131.89, 133.56, 134.03, 134.17, 139.52, 146.35, 149.11, 149.22 ppm; MS m/z 589.10 $(M+2)^{+}$; IR (KBr): $\tilde{\nu}$=3367, 3268, 1593, 1497 Cm^{-1}.

*4.3.12. 4-(3'-(4-chlorophenyl)-5-(4-methoxyphenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7l**)*

White crystal (64%): mp: 202°C; ^{1}H NMR (400MHz, DMSO) δ=8.378 (s, 1H), 7.81 (d, *J*=8 Hz, 2H), 7.77 (d, *J*=8 Hz, 2H), 7.70 (d, *J*=8.8 Hz, 2H), 7.56 (t, *J*=8.8 Hz, 4H), 7.44 (t, *J*=7.2 Hz, 2H), 7.27 (t, *J*=6.8 Hz, 1H), 7.01 (d, *J*=9.2 Hz, 3H) 6.97 (d, *J*=5.6 Hz, 3H) 5.65 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.02 (dd, *J*=12.4 Hz, *J*=5.2 Hz, 1H), 3.78 (s, 3H), 3.31 ppm (dd, *J*=17.6 Hz, *J*=4.8 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=43.15, 55.47, 55.79, 112.51, 114.60, 118.76, 122.81, 124.84, 127.02, 127.56, 127.76, 128.21, 129.23, 129.99, 130.22, 131.92, 133.41, 133.51, 139.53, 146.78, 149.04, 150.26, 160.73 ppm; MS m/z 584.10 $(M+H)^{+}$; IR (KBr): $\tilde{\nu}$==3417, 3278, 1587, 1499 Cm^{-1}.

4.3.13. *4-(1'-phenyl-3',5-di-p-tolyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7m**)*

Yellow crystal (62%): mp: 208°C; ^{1}H NMR (400MHz, DMSO) δ=8.28 (s, 1H), 7.80 (d, *J*=8 Hz, 2H), 7.66 (d, *J*=8 Hz, 4H), 7.55 (d, *J*=8.8 Hz, 2H), 7.42 (t, *J*=7.6 Hz, 2H), 7.31 (d, *J*=8 Hz, 2H), 7.25 (t, *J*=8 Hz, 3H), 7.00 (t, *J*=8.8 Hz, 4H) 5.63 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.07 (dd, *J*=17.4 Hz, *J*=12 Hz, 1H), 3.31 (dd, *J*=19.6 Hz, *J*=13.6 Hz, 1H), 2.35 (s, 3H), 2.31 ppm (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ=21.37, 21.48, 43.12, 55.58, 112.56, 118.64, 122.51, 126.59, 126.80, 127.40, 127.54, 128.38, 129.61, 129.71, 129.83, 129.95, 130.19, 133.54, 138.15, 139.43, 139.64, 146.66, 150.34 ppm; MS m/z 546.00 $(M-H)^{+}$; IR (KBr): $\tilde{\nu}$= 3365, 3247, 1592, 1497 Cm^{-1}.

*4.3.14. 4-(1',5-diphenyl-3'-(p-tolyl)-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7n**)*

White crystal (62%): mp: 198°C; ^{1}H NMR (400MHz, DMSO) δ=8.30 (s, 1H), 7.81 (d, *J*=8 Hz, 2H), 7.76 (d, *J*=6.8 Hz, 2H), 7.65 (d, *J*=8 Hz, 2H), 7.55 (d, *J*=8.8 Hz, 2H), 7.44-7.38 (m, 5H), 7.31 (d, *J*=7.8 Hz, 2H), 7.26 (t, *J*=7.8 Hz, 1H), 7.02-6.96 (m, 4H), 5.66 (dd, *J*=12.2 Hz, *J*=6.0 Hz, 1H), 4.09 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.34 (dd, *J*=15.6 Hz, *J*=11.6 Hz, 1H), 2.35 ppm (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ=21.37, 43.01, 55.00, 112.64, 118.64, 122.46, 126.60, 126.81, 127.46, 127.55, 128.41, 129.12, 129.73, 129.82, 129.95, 130.18, 132.35, 138.17, 139.63, 146.55, 150.26, 150.35 ppm; MS m/z 532.0 $(M-H)^{+}$; IR (KBr): $\tilde{\nu}$=3366, 3249, 1593, 1492 Cm^{-1}.

4.3.15. *4-(5-(4-methoxyphenyl)-1'-phenyl-3'-(p-tolyl)-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7o**)*

Yellow crystal (58%): mp: 178°C; ^{1}H NMR (400MHz, DMSO) δ=8.27 (s, 1H,), 7.81 (d *J*=8 Hz, 2H,), 7.70 (d, *J*=8.4 Hz, 2H,), 7.67 (d, *J*=7.6 Hz, 2H), 7.56 (d, *J*=8.8 Hz, 2H), 7.42 (t, *J*=7.6 Hz, 2H), 7.31 (d, *J*=8 Hz, 2H), 7.25 (t, *J*=7.2 Hz, 1H), 7.00-6.97 (m, 6H), 5.61 (dd, *J*=11.8 Hz, *J*= 6.4 Hz, 1H), 4.05 (dd, *J*=17.4 Hz, *J*=12.4 Hz, 1H), 3.76 (s, 3H), 3.31 (dd, *J*=17.4 Hz, *J*=6.4 Hz, 1H), 2.35 ppm (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ=21.37, 43.24, 55.54, 55.76, 112.43, 114.58, 118.63, 122.57, 124.91, 126.80, 127.37, 127.55, 128.21, 128.38, 129.84, 129.95, 130.20, 133.31, 138.16, 139.64, 146.78, 150.22, 150.29, 160.70 ppm; MS m/z 564.10 $(M+H)^{+}$; IR (KBr): *ν̃*= 3501, 3239, 1593, 1503, Cm^{-1}.

4.3.16. *4-(5-(3,4-dimethoxyphenyl)-1'-phenyl-3'-(p-tolyl)-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7p**)*

White crystal (62%): mp: 192°C; ^{1}H NMR (400MHz, DMSO) δ=8.25 (s, 1H), 7.81 (d, *J*=9.2 Hz, 2H), 7.67 (d, *J*=7.6 Hz, 2H), 7.53 (*J*=8.4 Hz, 2H), 7.42-7.38 (m, 3H), 7.32 (d, *J*=7.2 Hz, 2H), 7.25 (t, *J*=7.2 Hz, 2H), 6.98-6.95 (m, 5H), 5.62 (dd, *J*=11.6 Hz, *J*=6.8 Hz, 1H), 4.07 (dd, *J*=17.6 Hz, *J*=12 Hz, 1H), 3.82 (s, 3H), 3.77 (s, 3H), 3.35-3.31 (dd, 1H), 2.46 ppm (s, 3H); ^{13}C NMR (100 MHz, DMSO) δ=21.37, 43.28, 55.56, 56.06, 109.38, 111.95, 112.45, 118.64, 120.20, 122.60, 125.08, 126.81, 127.31, 127.52, 128.37, 129.85, 129.96, 130.19, 133.32, 138.16, 139.63, 146.68, 149.25, 150.40, 150.59 ppm; MS m/z 592.20 $(M-H)^{+}$; IR (KBr): *ν̃*= 3344, 3291, 1593, 1501Cm^{-1}. HRMS m/z: 594.213, 595.2159

4.3.17. *4-(3',5-bis(4-methoxyphenyl)-1'-phenyl-3,4-dihydro-1'H,2H-[3,4'-bipyrazol]-2-yl)benzenesulfonamide (**7q**)*

White crystal (54%): mp: 180°C; ^{1}H NMR (400MHz, DMSO) δ=8.27 (s, 1H), 7.80 (d, *J*=8 Hz, 2H), 7.71-7.67 (m, 4H), 7.55 (d, *J*=9.2 Hz, 2H), 7.42 (t, *J*=7.6 Hz, 2H), 7.25 (t, *J*=7.6 Hz, 1H), 7.05 (d, *J*=8.8 Hz, 2H), 7.00-6.95 (m, 6H), 5.60 (dd, *J*=12 Hz, *J*=6.4 Hz, 1H), 4.05 (dd, *J*=17.2 Hz, *J*=12 Hz, 1H), 3.79 (s, 3H), 3.78 (s, 3H), 3.31 ppm (dd, *J*=16.6 Hz, *J*=6.4 Hz, 1H); ^{13}C NMR (100 MHz, DMSO) δ=43.17, 55.54, 55.66, 55.78, 112.44, 114.59, 114.64, 118.57, 122.32, 124.92, 125.42, 126.72, 127.35, 127.54, 128.20, 129.81, 129.95, 133.30, 139.66, 146.78, 150.17, 150.25, 159.77, 160.70 ppm; MS m/z 580.10 $(M+H)^{+}$; IR (KBr): *ν̃*=3348, 3286, 1594, 1502 $Cm^{-1.}$.

5. Crystallographic analysis

The structure of compound **7o** was resolved by the PanalyticalX'Pert Pro Multipurpose Diffractometer (MPD). The instrument is a PANalyticalX'Pert Pro MPD, powered by a

Philips PW3040/60 X-ray generator and fitted with aX'Celerator detector. Diffraction data is acquired by exposing powder samples to Cu-Kα X-ray radiation, which has a characteristic wavelength (λ) of 1.5418 Å. X-rays were generated from a Cu anode supplied with 40 kV and a current of 40 mA. The data were collected over a range of 5.0084 to 49.9904 ˚2θ with a step size of 0.0170 ˚2θ and nominal time per step of 25.1954s, using the scanning X'Celerator detector (hence the counting time per step). Fixed anti-scatter and divergence slits of 0.4354˚ were used together with a beam mask of 10mm and all scans were carried out in 'continuous' mode. Phase identification was carried out by means of the X'Pert accompanying software program PANalytical High Score Plus in conjunction with the ICDD Powder Diffraction File 2 Database (1999), ICDD Powder Diffraction File 4 - Minerals (2012), the American Mineralogist Crystal Structure Database (March 2010) and the Crystallography Open Database (February 2012; www.crystallography.net) . Crystallographic data for the structure **7e** have been deposited with the Cambridge Crystallographic Data Center (CCDC) under the number CCDC- 1475330. Copies of the data can be obtained, free of charge, on application to CCDC 12 Union Road, Cambridge CB2 1EZ,
UK [Fax: +44-1223-336033; e-mail: deposit@ccdc.cam.ac.uk or at www.ccdc.cam.ac.uk]

6. Biological Studies:

6.1. In-vitro anti-malarial activity

The *in vitro* antimalarial activity was performed by reported method [19]. Briefly the parasitized blood was made by infecting red blood cells with CQS (3D7) strain and CQR (RKL9) strain of *P. falciparum* separately with 2-3% parasitemia and 8% hematocrit in RPMI 1640 medium supplemented with human AB+ serum. Parasitized blood was added to the wells of 96 - well plate containing 100 µl of test sample (1 mg/ml) diluted in medium at different concentrations. After incubation of the plates at 37ºC for 24 hours the blood smear was prepared from all wells and stained with giemsa stain. Schizonts stage (3 or more merozoites containing) were counted and result was analysed using dose-response curves by non-linear regression analysis using HN-NonLin regression analysis.

6.2. In-vivo antimalarial activity

The *in vivo* antimalarial efficacy was determined by Peter's 4-day suppressive test [16, 19] by using *P. berghei* NK65 strain and female Swiss albino mice (20-25 g of body weight). Briefly animals were divided into five groups of five mice each. The animals were inoculated

intraperitonially with approximately 10-15% of *P. Berghei* infected erythrocytes from a donor mouse. Compound **7o** was administered orally at a dose of 25 mg/kg body weight and 50 mg/kgbody weight after four hours of infection. Chloroquine (5 mg/kg body weight) was administered to positive (standard) control group. Negative control group (5 mice) were administered the same amount of solvent used to suspend the test compound. The compounds were administered on day 0, 1, 2 and 3. On day 4, blood was taken from tail vein, smears were made, fixed with methanol and stained with Giemsa and analysed for parasitemia microscopically (1000 magnification). The percent parasitemia and average percent suppression of parasitemia were determined and compared to negative control group. The animals were observed for 30 days and mean survival days (MSD) was determined by calculating the death of mice during 30 days observation period.

6.3.Mechanistic studies (heme binding studies)

*6.3.1. Binding of compound **7o** with monomeric heme:*

The study was performed by reported method [16]. The working solutions of hemin were prepared by mixing 20 µl stock solutions (1.2 mM) with 4 ml DMSO and 1 ml 0.2 M HEPES buffer (pH 7.4). The final volume was made up to 10 ml with double distilled deionized water. The increasing concentration (0-100 µM) of **7o** solution in DMSO was used for titration of heme (2.4 mM) and absorbance was recorded at 402 nm. The same procedure was followed for conducting the experiment at pH 5.6, except that 2-[N-morpholino] ethanesulphonate (MES) buffer (pH 5.4) was used in place of HEPES buffer.

*6.3.2. Binding of **7o** with dimeric heme*

Heme stock solution (10 mM) was prepared by dissolving hemin chloride in 0.1 M NaOH and sonicated for 30 min. Heme solution (60 mM) was prepared by diluting stock solution with 20 mM phosphate buffer (pH 5.8). Compound **7o** stock solution (2 mM) was prepared in DMSO. Titrations were done by addition of stock solution of compound **7o** to 60 mM heme solution and absorbance was recorded at 362 nm.

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Conflict of interest

The authors report no declarations of interest.

Supplementary data: The supporting information includes spectral data details (NMR, IR and Mass) and detailed procedures of some biological activities.

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Table Captions

Table 1: *In-vitro* anti-malarial activity studies of synthesized compounds (**7a-7q** and **8, 9a-d)** against CQ^S 3D7 and CQ^Rstrain RLK-9 of *P. falciparum*

Table 2: *In vivo* antimalarial activity of **7o** in mice infected with *P. berghei*

Table 3: Binding constants for compound **7o** and chloroquine with heme

Figure Captions

Figure1: Reported antimalarial compounds acting via inhibition of hemazoin/β-haematin formation

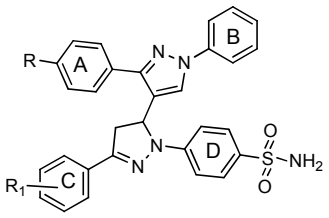
Figure 2: *trans* conformation of the compound **7e** identified from crystal structure. The same is deposited in Cambridge Crystallographic Data Centre (CCDC) under the number CCDC-1475330.

Figure 3: a) Titration of compound **7o**with monomeric heme at pH 5.6; **b)** Titration of compound **7o** with monomeric heme at pH 7.4

Figure 4: Job's plot of monomeric heme complex formation with compound **7o**; **a)** at pH 5.6; **b)** at pH 7.4; X (mole fraction of compound **7o**) = [compd. **7o**]/[compd. **7o**]+[heme]; A_0 is the absorbance, when x = 1 and A is the absorbance at respective values of x

Figure 5: a) Titration of compound **7o**with dimeric heme at pH 5.8; **b)** Job's plot of dimeric heme complex formation with compound **7o** at pH 5.8

Table 1: *In-vitro* anti-malarial activity of synthesized compounds (**7a-7q** and **8, 9a-d)** against CQ^S (3D7) and CQ^R (RLK-9) strains of *P. falciparum*



7a-q

Cpd	R	R_1	3D7 (EC_{50}) µg/ml	3D7 (EC_{50}) µM	RLK-9 (EC_{50}) µg/ml	RLK-9 (EC_{50}) µM	RI
7a	H	2,4- diCl	2.694±0.054**	4.58	NT	NT	--
7b	H	4-Cl	1.858±0.013**	3.35	NT	NT	--
7c	H	4-F	3.579±0.022**	6.67	NT	NT	--
7d	H	4-OH	1.629±0.003**	3.05	NT	NT	--
7e	H	4-OCH_3	0.781±0.003**	1.42	0.98±0.042**	1.78	1.25
7f	4-F	4-Br	1.908±0.003**	3.08	NT	NT	--
7g	4-F	4-F	3.538±0.034**	6.36	NT	NT	--
7h	4-F	4-CH_3	1.227±0.005**	2.23	NT	NT	--
7i	4-F	4-OH	0.868±0.006**	1.57	1.12±0.036**	2.02	1.29
7j	4-F	4-OCH_3	0.941±0.006 ns	1.65	1.36±0.067**	2039	1.44
7k	4-Cl	4-Cl	1.651±0.004**	2.80	NT	NT	--
7l	4-Cl	4-OCH_3	0.941±0.007 ns	1.61	1.24±0.053**	2.12	1.31
7m	4-CH_3	4-CH_3	0.884±0.009**	1.43	0.9±0.047**	1.64	1.14
7n	4-CH_3	4-H	1.641±0.012**	2.97	NT	NT	--
7o	4-CH_3	4-OCH_3	0.781±0.006**	1.38	0.74±0.072**	1.31	0.94
7p	4-CH_3	2,3- diOCH_3	0.945±0.014 ns	1.61	0.92±0.051**	1.55	0.97
7q	4-OCH_3	4-OCH_3	1.315±0.002**	2.26	NT	NT	--
CQ			0.95	2.97	3.60	11.25	3.78

Cpd: compound; NT: not tested; RI (Resistance Index) = IC_{50}:*Pf*RKL9/IC_{50}: *Pf*3D7; ns non-significant compared to control;** significant at P<0.01compared to control.

Table 2: *In vivo* antimalarial activity of **7o** in mice infected with *P. berghei*

Compound	dose (mg/kg/day X 4)	Route	% reduction parasitemia (day 4)	Mean survival days (MSD)
7o	25	po	68.42	13.4
7o	50	po	82.49	19.8
CQ	5	po	97.10	30
Negative Control	-	-	-	7.2

Table 3: Binding constants for compound **7o** and chloroquine (**CQ**) with heme

	Monomeric heme (Log K ± σ)		**μ-oxo-heme (Log K ± σ)**
Compound	**pH 5.6**	**pH 7.4**	**pH 5.8**
7o	5.58	5.12	5.19
CQ	4.72	5.23	5.68
Stoichiometry	1:1	1:1	1:1

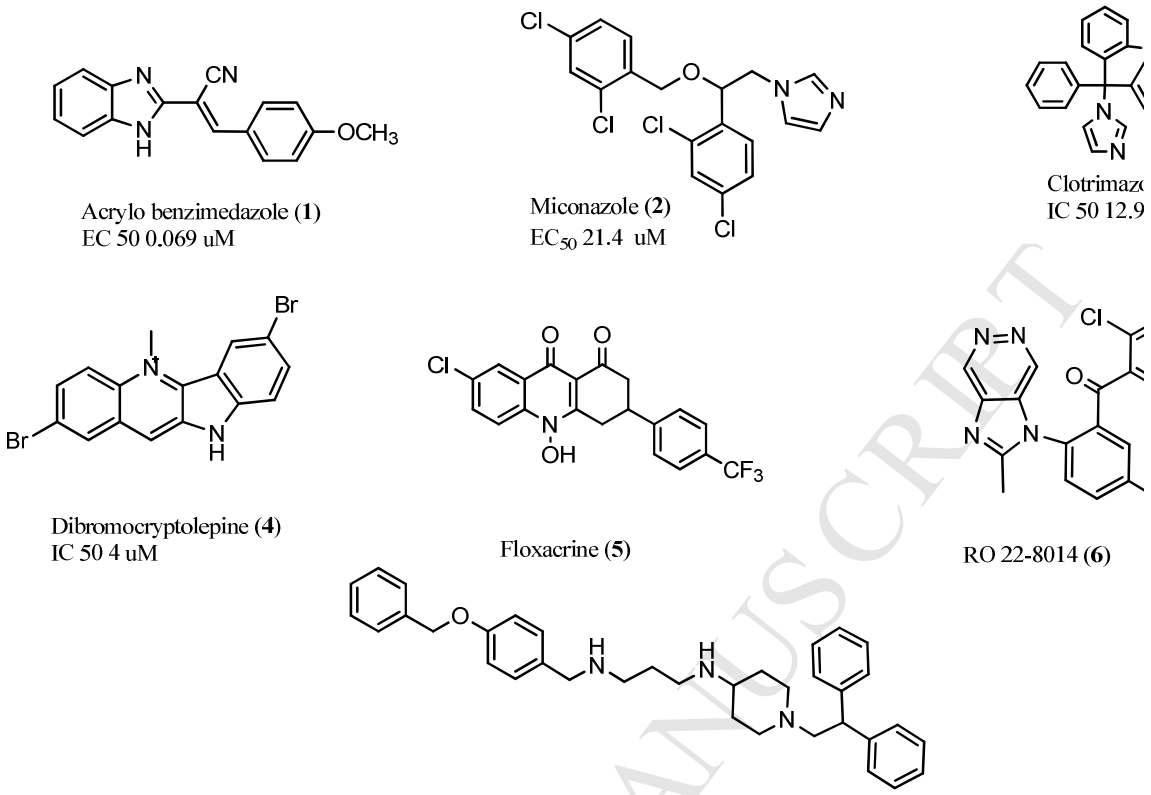
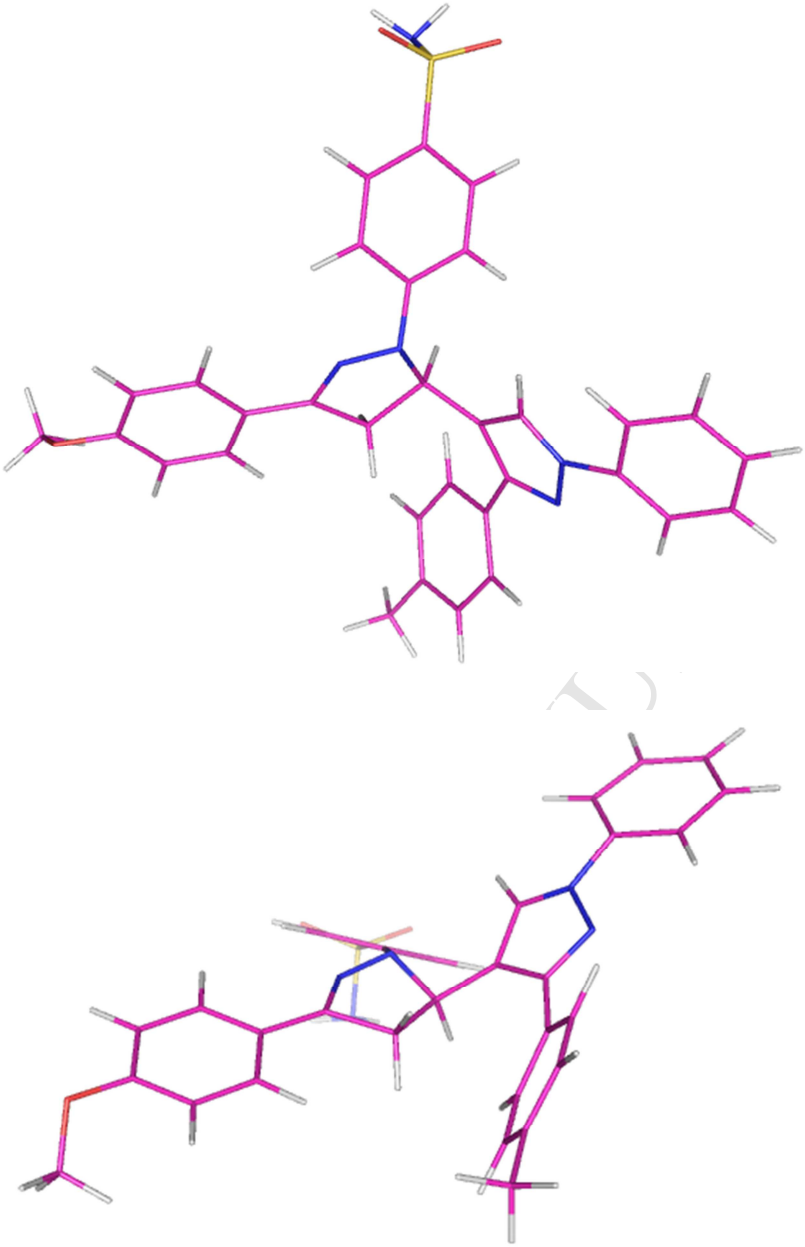


Figure1: Reported antimalarial compounds acting via inhibition of hemazoin/β-haematin formation



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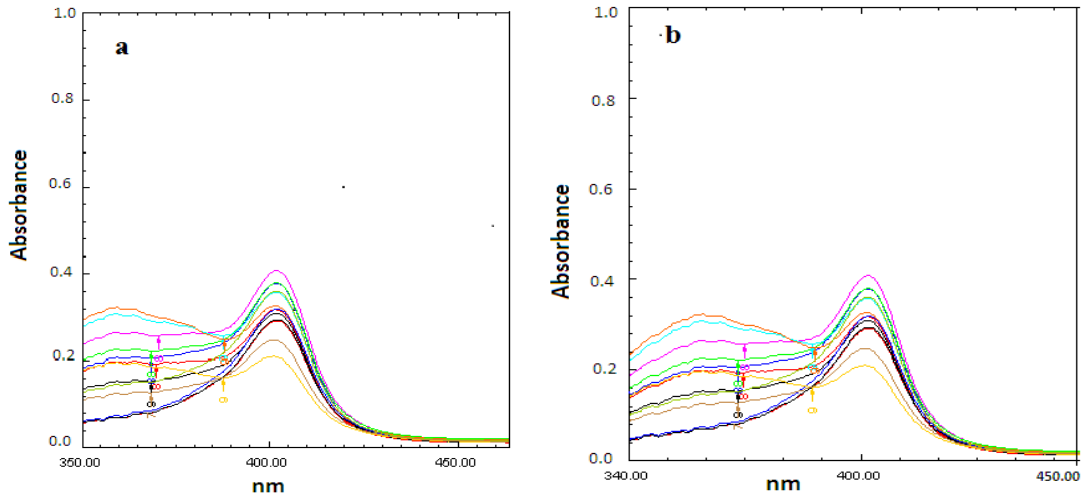

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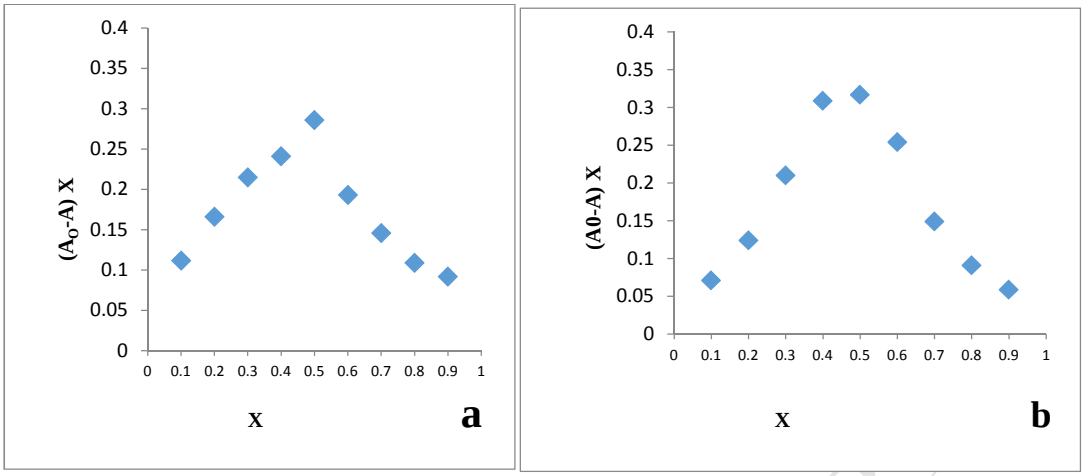

Figure 4. Job's plot of monomeric heme complex formation with compound **7o**; **a)** at pH 5.6; **b)** at pH 7.4; X (mole fraction of compound **7o**) = [Compd. **7o**]/[Compd. **7o**] + [heme]; A_0 is the absorbance, when x = 1 and A is the absorbance at respective values of x

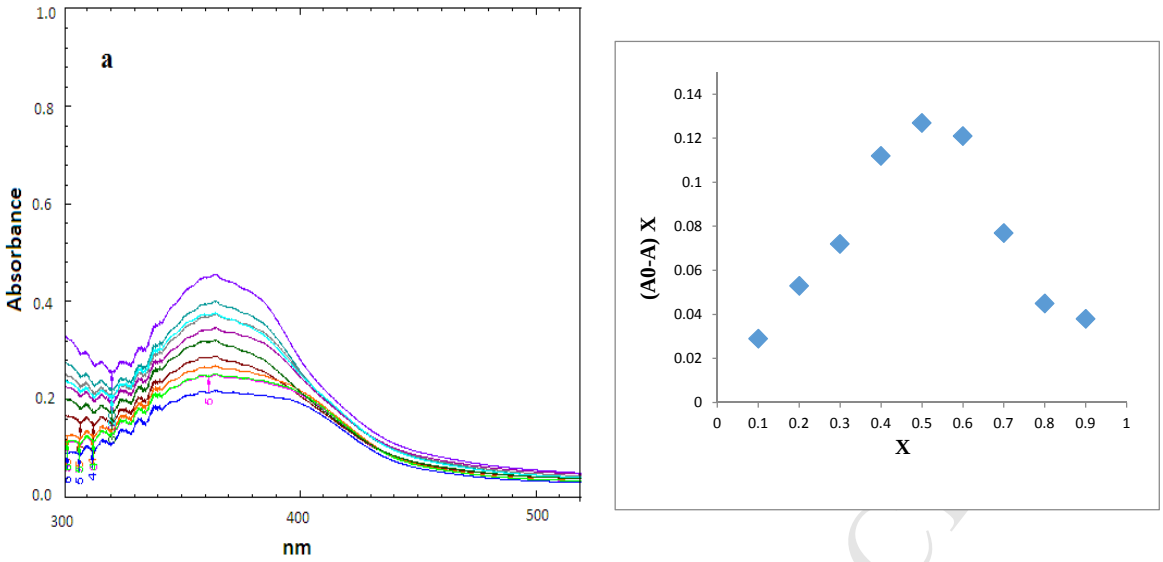

Fig. 5 a) Titration of compound **7o** with μ-oxodimeric heme at pH 5.8; **b)** Job's plot of dimeric heme complex formation with compound **7o** at pH 5.8

Pyrazole-Pyrazoline as Promising Novel Antimalarial Agents: A Mechanistic Study

Highlights

- A series of pyrazole pyrazoline endowed with benzenesulphonamides have been synthesized.
- All the synthesized compounds screened showed good antimalarial activity.
- Most potent analogues **(7e** and **7o)** exhibited promising antimalarial activity against CQ^S (3D7) strain with IC_{50} of 0.781 and 0.781µg/ml respectively.
- Compounds **7e** and **7o** was active against CQ^R (RKL9) strain with IC_{50} of 0.0.98 and 0.74 µg/ml respectively
- Compound **7o** was also found to show 82.49% reduction in parasitaemia (day 4) in *P. berghei* mouse model.
- The compounds showed good hematin binding affinity indicating their possible mechanism of action